A SYMBIOTIC FUNGUS OCCURRING IN THE FAT-BODY OF *PULVINARIA INNUMERABILIS* RATH.[1]

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During the late winter and spring of 1920 the present writers became interested in the supposedly symbiotic organisms which occur in various scale insects, hoping that they might be able to propagate some of them in artificial cultures and learn something of their physiological activities. From knowledge gained thus, it seemed probable that they might be able better to determine whether such organisms exist in the insects as true symbionts, as mere commensals or as innocuous parasites.

As is well known through the investigations of several workers, the entrance of the symbionts[2] into the egg of scale insects can be readily followed as well as their behavior during embryological development. A good account of this has been given by Shinji ('19) who also includes a summary of the previous work of other authors.

In the nymphal or full-grown scales of many species it is more difficult to find and interpret the symbionts and it seems probable that in some cases they must either become very much reduced in numbers, very highly modified, or perhaps reduced to minute and unrecognizable spores or granules.

Several workers who have recently examined the symbionts of Coccids (*e.g.,* Buchner '12, Sulk '10, Teodoro '18) regard them as yeasts (Saccharomycetes), although Berlese ('06) referred one species to the genus *Oöspora,* one of the *Hyphomycetes.*

Before beginning our work with *Pulvinaria innumerabilis,* the cottony maple scale, we examined several other species of Coccids, but were unable successfully to cultivate the symbionts from these, with the possible exception of one of the pine scales,

[1] Contribution from the Entomological Laboratory of the Bussey Institution, Harvard University, No. 176.

[2] We have used this name as a convenient designation already in current use and will discuss its appropriateness on a later page.

Chionaspis pinifoliæ Fitch. From the latter we isolated an organism, perhaps related to *Oöspora,* but we could not secure it with sufficient regularity to satisfy ourselves that it was really the symbiont, and not a contaminating species of microörganism of which one always encounters numerous species in work of this kind. Just how closely related the symbionts of various Coccids may be, must remain a matter of doubt until a considerable number have been carefully investigated, and preferably cultivated also, but our own observations lead us to think that more than one type of organism will be found after careful, systematic study.

In the following brief review of literature we have considered only such contributions as appear to bear directly on symbionts quite probably closely related to the form with which we have worked.

The first reference to the occurrence of symbiotic organisms in Coccidæ is that of Leydig ('54) who found discrete, lanceolate bodies which he believed to lie free in the lymph of *Coccus* (now *Lecanium*) *hesperidum.* He described them as 4 μ in length, multiplying by buds which do not separate till they have attained the size of the parent cell. Neither at this time, nor in 1860 in his contribution to the development of the Daphnids, did he realize their significance. From the rather large size and method of multiplication, these are probably similar to the organisms found in *Pulvinaria,* which also appear in freshly mounted smears as though they were free in body fluid. Putnam ('79) studied in considerable detail the biology, anatomy and development of *Pulvinaria innumerabilis* in Iowa. In connection with a section devoted to the contents of the ovaries he gave an account of the organisms with which we deal in the present paper. His observations were so carefully made that we have thought it worth while to include the following resumé. On opening a female at any time from October to May, five classes of bodies are set free, all of them apparently associated with the development of the eggs. These are: First, a clear protoplasmic liquid; second, clear spherical globules 10 μ–30 μ in diameter, lighter than water, and not taking the ordinary aniline stains. He was undoubtedly cor-

rect in believing these to be yolk and fat globules. Third, exceedingly minute, apparently spherical bodies, heavier than water and staining with eosine. Putnam thought that these might be bacteria although he suggests that they may be comparable to the blood-disks [erythrocytes] of vertebrates or that they may be stages in the development of the fourth class of bodies which he next considers. That all of these suppositions are probably incorrect will appear from our account on a later page. Fourth, small oval bodies 3 μ–5 μ in diameter and 10 μ long and heavier than water. These represent the organism which we have studied but Putnam was unable to decide whether they were spermatophores or whether they corresponded to the pseudonavicellæ observed by Leydig in *Lecanium,* as had been suggested to him by Dr. E. L. Mark.[1] A fifth class of bodies observed were the small incompletely formed eggs.

There can be no doubt that Putnam found the fungus with which we have worked as his description and figures make this point very clear. As he found it in all cases, it is further clear that the symbiont enjoys a wide range since his observations were made on specimens collected in the middle west and our own on material from eastern Massachusetts.

Metschnikoff ('84) found in a crustacean, *Daphnia magna,* a fungus which he called *Monospora bicuspidata.* This he regarded as a parasite, but recent developments in the study of apparently similar organisms in insects, suggests that these Crustaceans should be reëxamined.

Moniez ('87) described a fungus which he called *Lecaniascus polymorphus,* occurring in the scale insect, *Lecanium hesperidum.* He refers to Leydig's 1854 paper, mentioning the fact that *Lecaniascus* is evidently the same as Leydig's pseudonavicellæ. Moniez speaks of the organism as a parasite, and he found it in all specimens, both young and old, of the *Lecanium* that he examined. He described the isolated cells as 4–5 μ in length, and found mycelia attaining a length of 50–60 μ. Some doubt is cast upon this author's conclusions by Vejdovsky ('07) who suggests that Moniez may have seen two microörganisms, one represented

[1] Mark ('77) does not consider these bodies, however, in his paper on the anatomy and histology of the Coccidæ.

by the single cells and another by the mycelia and asci, the latter perhaps *Alternaria tenuis,* a parasitic fungus that attacks various Coccids of the genus *Lecanium.*

Lindner (1895) found in an European scale insect (*Aspidiotus nerii*) a yeast-like organism which he regarded as related to *Saccharomyces apiculatus.* By crushing one of the insects between a slide and cover-glass he observed large numbers of the yeasts, both between the small masses of fat-body and actually in the adipocytes. The organisms he described and figured as usually very long, pointed at one end, or lanceolate, sometimes joined in pairs by their acute tips, and frequently budding after the manner of yeast cells. At that time Lindner was unable to cultivate the organism although he evidently regarded it as a parasite, naming it *Saccharomyces apiculatus,* var. *parasitus.* He found it forming a mass at the posterior pole of the egg and made an ingenious explanation for its presence there, suggesting that one of the pointed tips perforated the egg and gave off a bud which then multiplied to form the mass or mycetome.

A later paper by Lindner ('07) which appeared in the *Wochenschrift für Brauerei* we have not seen, but from published reviews of this, it appears that it contains nothing bearing on the physiology or systematic position of his yeast-like organism.

Berlese ('06) studied in detail an organism which he found in *Ceroplastes rusci,* and was successful in growing it on artificial media. From Berlese's account, it appears that the fungus, which he calls *Oöspora saccardiana* is very similar to the one described by us in the present paper. The yeast-like cells in the Coccid vary in length from 4–12 μ, sometimes attaining a length of 16–18 μ in early summer, agreeing in size and also in form with those we have observed in *Pulvinaria.* In culture there is a great similarity in the general morphology and development of mycelia, and although Berlese gives few details concerning cultural characteristics, at least one statement shows a striking difference between the two. He found that although the symbiont from *Ceroplastes* grows rapidly on gelatine media, that these are not liquified, while as will appear from our account, the *Pulvinaria* fungus exhibits a powerful liquefactive action on gelatine.

Concerning the distribution of the symbionts in the body of *Ceroplastes,* Berlese gives no account, except to state that they generally occupy the visceral cavity completely in all individuals, in numbers estimated at from 60,000–70,000 cells.

Two other genera of soft scales, *Kermes* and *Physokermes,* have been the subject of investigation by Sulc ('07) and Vejdovsky ('07). The former found two distinct symbiotic organisms in two species of these Coccids, readily distinguishable from one another on the basis of size and form. These he described as representatives of a new genus, *Kerminicola.* Vejdovsky regarded them as Saccharomycetes in which opinion Sulc concurred. The microscopical structure of the symbionts was carefully described and is similar to that of the species in *Pulvinaria* studied by us, although *Kerminicola* evidently has a much more prominent and discrete nucleus and fewer multinucleate cells; also the form of the cells is generally much more elongate. Vejdovsky found the symbionts in the fat cells of the host in large numbers and observed them freed in the hæmolymph as a result of a disintegration of the adipocytes which he believed due to the activity of the included organisms. He, therefore, regarded them as parasites, but pointed out that their activities do not affect the gonads of the host, nor the development of the eggs in its body. They do, however, serve to break down the fat and to consume the remaining tissues of the host's body, after which it remains a shell for the protection of the now fully developed nymphal *Kermes.*

In a later paper, Sulc ('10) gives a more extended account of the similar organisms of a number of Homoptera and speculates at considerable length upon the relations between the insects and fungi. After more extended study, his ideas have been considerably modified and he has come to regard the microörganisms as essentially symbiotic in their association with their host insects. He suggests that the production of enzymes by yeasts (for he still refers the symbionts to the Saccharomycetes) must be considered in any interpretation of their physiological relations to the insects. Apparently he made no attempts at cultivation *in vitro.*

Pierantoni has considered the symbionts of certain Coccidæ in

several papers of which that of 1910 in the *Zoologischer Anzeiger* is of greatest interest in the present connection. In *Icerya purchasi,* he traced the entrance of the organisms into the egg of the scale insect and its subsequent behavior through the formation of the polar mass in the egg to the development of the mycetocyte in the larva. He found that the individual symbionts of this species were at first round or oval, and not noticeably elongated, and that later during embryonic development and at the time of hatching they became quite inconstant in form, varying from rounded or oval to much elongated and frequently strongly curved cells, all, however, of about the same diameter. These long forms may fragment, each piece becoming a new individual, while the short ones commonly divide by fission into equal parts. Occasionally, however, Pierantoni found cells multiplying by budding in the body cavity of the host, and more rarely in the mycetome.

On a gelatine medium, with high sugar content (20 per cent. saccharose) he was able to cultivate a yeast-like organism from the mycetocytes. These developed after four days' incubation as colonies that are described as small spheres in the gelatine which develop a sort of finger-like process which becomes prolonged toward the surface of the gelatine and then emerges projecting above it in the form of a finger, or with the base enlarged and pyriform. The individual organisms were of yeast-like form with buds more or less developed. It thus appears that the organism obtained in culture by Pierantoni differed greatly in form, size, and method of multiplication from the organisms in the insects. as will be shown later, the cultures obtained by us from *Pulvinaria innumerabilis* exhibit no such remarkable distinctions, in morphology and reproduction, from the cells in the host insect. Although he makes no mention of the development of mycelia in his cultures, the form of the colonies indicates without question that such must have been present, and that if the organisms in the mycetome were actually those obtained in culture, the symbiont of *Icerya* is a true fungus.

The Symbionts of the Half-grown Pulvinaria.

In early April the overwintered cottony maple scale-insects are partly grown and may be found attached to the bark of small twigs on the food plants, which consist of various maples and a few other woody plants. At this time they are nearing the end of their period of hibernation and do not yet exhibit any active growth.

If a specimen in this condition be crushed on a slide in normal salt solution, the symbionts may be readily seen free in the liquid. They are heavier than the medium and fall next to the slide, thus separating from the released fat globules which accumulate above, against the cover-slip. In such a preparation all the organisms appear to be in the liquid, as those in the fat cells are not readily discernible on account of their hyaline nature. This, no doubt, accounts for the statements that the symbionts occur in the lymph rather than in the tissue.

In sections it is evident, however, that the organisms are absent, or at least very nearly so, from the blood and that they are very generally distributed through the fat body, imbedded in the adipose cells. They are usually spaced in a quite regular way showing that they migrate or at least change their position in the cells subsequent to multiplication. The density of distribution is well indicated in the drawing (Fig. 1, *A*) which is made from a section of 6 μ in thickness where all of the symbionts present have been sketched. The photograph on Plate I., Fig. 1, is made from a typical cross-section through an entire insect, in which the symbionts appear as minute oval dots. On Plate I., Figs. 2, 3 and 4, are reproduced several small areas of the fat-body viewed at higher magnification with their symbiont inclusions. Sketches of a few still more highly magnified symbionts are shown in Fig. 1, *B*. Here it will be seen that they are extremely variable in size and shape, but always quite distinctly oval in form with one pole more acute and the opposite one more rounded. They vary in length from 10–16.7 μ by 5–6.5 μ in width, with an average size of 10–12.5 μ by 5.7 μ. Budding forms are frequently present, the bud developing at the narrow pole and separating either as a small oval, or more rarely rounded, cell. The buds at the time of sepa-

ration are of the same general shape as the cells from which they originate, but much smaller, varying in length from 6.2–6.7 μ. Some buds are nearly round, in which case they separate when considerably smaller, about 3.7 μ in diameter.

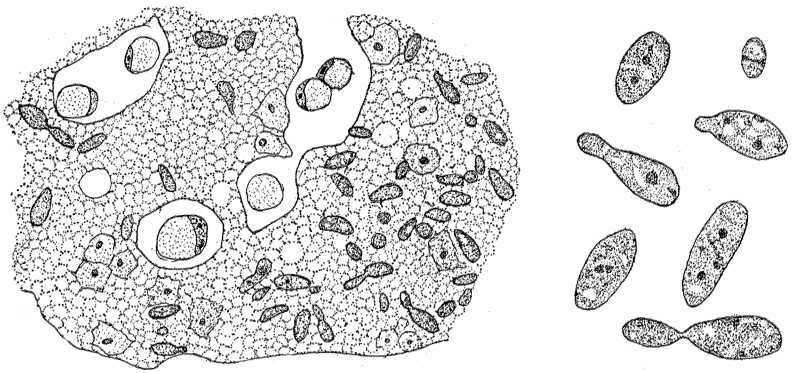

FIG. 1. *a*, Section of fat body showing symbionts *in situ* in overwintered nymphal *Pulvinaria*, × 260; *b*, isolated symbionts, stained with methylene blue and eosin, × 1,000.

The internal structure of the symbionts shows very little uniformity. Sections of tissue fixed in Zenker's solution and stained with methylene blue and eosin show one or several rather distinct deeply stained portions that resemble nuclei. When one is present it is usually central, when two or more are present, they are separated rather evenly from one another and from the cell wall. The remaining protoplasm shows irregular denser streaks and spots of irregular size, with usually one large, several smaller, or one large and one small vacuole, with generally a number of minute clear spots that can be seen only after very close examination. We have tried to bring out other details of structure by different methods of fixation and staining but without much success. Smears fixed by such methods as drying and submersion in absolute alcohol and subsequent staining with Giemsa's stain, Manson's Protozoan stain and carbol-fuchsin, give quite similar pictures to those in sections which are disappointing from a cytological standpoint. Evidently the symbionts are of very delicate consistency. No chains of symbionts occur at this time (early April) except for the single attached buds, and no masses of contiguous ones are to be found.

Changes in the Symbionts after Hibernation.

Our observations on these are very fragmentary as our available time at this season was occupied with the cultural experiments described below. It appears quite certain, however, that the oval symbionts of the early spring *Pulvinaria* nymphs undergo further multiplication and morphological changes in late April, and during May. A cursory examination of specimens about May 10 showed the symbionts in groups sometimes forming short strings, somewhat similar to the typical mycelium which was obtained in cultures. The picture at this time indicates quite clearly the fungous nature of the yeast-like organisms just described, found before spring growth begins.

Isolation of the Fungus from Pulvinaria.

Our first attempts to cultivate in artificial media the yeast-like cells present in *Pulvinaria* were made in early April. The overwintered, partly grown scales were brought into the laboratory still attached to the maple twigs on which they occur. The scales can readily be detached from the twig by means of a sterile needle and allowed to fall upon a sterile microscope slide. The scales thus removed, were treated in several ways as experience had taught us that material of this sort is very apt to be contaminated on the surface by various microörganisms. Some were treated with 85 per cent. alcohol for a few minutes, others rapidly passed through the flame of a Bunsen burner, and others were used without treatment, except to avoid contact with any unsterile object. Our first media were potato agar and "sugar agar" which consisted of potato agar to which varying amounts of maple sugar (2½ per cent.– 20 per cent.) had been added. These tubes are readily inoculated by crushing the scale insect between two microscope slides and streaking the agar with a loop dipped in the body-juices thus extracted. It is also easy to drop the whole insects into culture tubes and then crush them on the agar by means of a dissecting needle. From a large series of tubes inoculated according to these methods, nearly one half showed after three days a good growth, white in color, spreading on the surface of the media. These colonies appeared to de-

velop almost equally well in the greatly diverse concentrations of sugar and in the plain potato agar. A microscopic examination at this time showed that nearly all the tubes in which growths occurred contained the same microörganism, and that only a few were contaminated by molds (*Penicillium*) and bacteria. The abundant species showed large numbers of budding yeast cells like those in the living scale insects and the development of mycelium as well showing that the symbiont was a fungus and not a yeast as one might otherwise be led to believe from a study of the living insects, at this season of the year when only single budding cells occur in the fat body.

Several of the colonies thus obtained were plated, found to be pure and sub-cultures were then made from these which have furnished the material for the description and cultural characters detailed below. Although the morphology of the organism in the living insects and in the cultures and the fact that it was recovered in such a large proportion of the cultures, left little doubt as to the identity of the two, we undertook some serological tests to corroborate if possible the conclusion based on morphological data.

For this purpose, two rabbits were secured, inoculated with bouillon cultures of the fungus, and serum from each was tested with the cultures and also with the organisms in the living scale insects. The following table shows the doses used and the reactions of the rabbits.

Date.	Amount of Culture.	Weight.	
		Rabbit A.	Rabbit B.
April 13	3 c.c.	2185 gms.	1905 gms.
April 16	5 c.c.	2180 gms.	1910 gms.
April 20	5 c.c.	2080 gms.	1910 gms.
April 24	6 c.c.	2110 gms.	1895 gms.
April 29	6 c.c.	2030 gms.	1915 gms.

One week later some blood was withdrawn from each rabbit and serum prepared. A precipitin test with the culture gave a positive reaction after four hours, and agglutination was very pronounced after one and one half hours as examined under the microscope. On account of the impossibility of securing sufficient material from the living scales, it was possible to try with

them only the agglutination test. With these the reaction was not so pronounced as with the yeast-like forms in culture, but nevertheless distinctly positive. Unfortunately by the time the animals had been immunized (early May) the number of yeast-like cells in the insects had decreased and the reaction could not be so readily observed as in the cultures, or so well as it might have been several weeks earlier in the spring when the insects contained innumerable, separated, oval cells. The data from the rabbit experiments has, however, convinced us that there can be practically no question that the organism cultivated is actually the one present in the insects. Furthermore, since we have never failed to observe it in living scales from this locality, and since Putnam (v. *antea,* p. 301) found it invariably present in Iowa, it is undoubtedly present regularly in *Pulvinaria innumerabilis.*

Cultural Characteristics of the Fungus.

As stated at the outset, we wished to grow the symbiont on artificial media, not only to describe it adequately, but to determine as completely as possible its physiological activities. In order to do this, sub-cultures from the original isolations were planted upon various media, such as are in general use by bacteriologists and mycologists. From these, the following observations were made.

Growth on Solid Media.

Potato Gelatine Colonies.—Growth rapid; after 72 hours, cottony with flocculent elevated center and filamentous edge, diameter of center 1 mm., width of margin 1 mm. Liquefaction cup-shaped.

Nutrient Gelatine Colonies.—Growth slow; after 72 hours, rounded, with central elevation. Diameter 0.3 mm., with roundly lacerated edge. Liquefaction cup-shaped.

Potato Agar Colonies.—Growth rapid; after 72 hours, filamentous, ciliate (sub-surface) or rounded (surface). Disk when present, smooth; elevation convex; edge of round colonies smooth, that of irregular colonies radiately filamentous or ciliate. Internal structure finely granular. Diameter 1.5–2.5 mm.

Nutrient Agar Colonies.—Growth slow; after 72 hours, round,

with smooth surface and convex elevation. Edge smooth; internal structure finely granular, with irregular central core. Diameter 0.6 mm.

Potato Gelatine Stab.—Liquefaction begins in 48 hours; growth best at tip, the line of puncture filiform; liquefaction at first napiform, becoming stratiform, surface umbonate. Medium unchanged.

Nutrient Gelatine Stab.—Same, but liquefaction proceeds more slowly and is more nearly crateriform.

Potato Agar Slant.—After 72 hours growth is abundant, spreading, densely rhizoid, convex. Color white, opaque, surface glistening, smooth. No odor, consistency viscid. Medium unchanged.

Nutrient Agar Slant.—After 72 hours growth is moderate, beaded, with a few rhizoid colonies where medium is thin. Surface smooth, glistening. Color white, opaque. No odor, consistency butyrous. Medium unchanged.

Locke's Agar Slant.—Growth like other agar slants, scanty, beaded, many rhizoid colonies, consistency butyrous. After a much longer incubation (four weeks) there is not a very heavy growth, but it is still more or less beaded and highly rhizoid on the sides and extending deep into the agar.

Molisch's Agar Slant.—After 72 hours growth abundant, much like that on potato agar. Consistency very ropy. No pigment, even in old cultures.

Potato Agar (with Yeast[1]*) Slant.* Growth after 72 hours abundant, like that on potato agar, but with fewer rhizoids.

Potato (Pieces).—Colonies raised, slightly yellow, growth good. There is a trace of ammonia.

Growth in Liquid Media.

Nutrient Bouillon.—After 48 hours growth good; after 72 hours, no surface growth, no clouding and no odor, but with an abundant viscid sediment. No hydrogen sulphide is produced.

Locke's Solution.—After 48 hours not very much growth; after 72 hours, no surface growth, no clouding and no odor, but with an abundant viscid sediment. After a much longer incuba-

[1] Made by adding 2 per cent. of thoroughly crushed bread yeast.

tion at room temperature (four weeks), the growth becomes very flocculent and adheres to the surface of the glass where it is finely dotted with very dark, pigmented spots.

Molisch's Solution.—After 48 hours rather good growth; after 72 hours, no clouding or surface growth, but with very abundant, slightly viscid sediment, no odor.

Sugars.—We have grown the organism in six sugars: lactose, dextrose, mannite, saccharose, levulose and maltose. None of these, however, furnish any differentiating characters; in all there is good growth without the formation of gas, but with heavy viscid sedimentation, and after prolonged incubation, the development of a distinct surface film.

Milk.—Growth is abundant, and after four to six days incubation the milk begins to clear at the top, sediment collecting in the lower half of the liquid. Litmus milk becomes distinctly red at the time of clearing. After about a week, the liquid becomes whey, with a sediment at the bottom of the tube.

Anaërobic Media.—We have not been able to cultivate the organism under anaërobic conditions.

Production of Enzymes.

Protease.—Gelatine is rapidly liquefied. Milk cultures, tested after 20 days, give a positive reaction with $MgSO_4$, NaOH and $CuSO_4$, showing the presence of peptones.

Lipase.—After both 20 and 30 days' incubation, cultures in either whole or skimmed milk give positive reactions. There is a strong odor of butyric acid. Ethyl butyrate is also decomposed with the formation of butyric acid. The 30-day culture was tested with pyrogallol and stannic chloride and gave a positive reaction also.

Diastase.—Bouillon cultures after ten days' incubation were treated with starch paste at incubator temperature for 48 hours; after this, Fehling's solution was reduced, demonstrating the presence of sugar.

Morphology of the Cultivated Fungus.

The cultures show during the first few days only yeast-like budding cells like those seen in the early spring in the fat-body

of the insects. These vary considerably in size, ranging from 6–16 μ in length and from 3–9 μ in width. They are thus more variable in size in culture than in the insect and generally more elongate. The maximum length is about the same, but there are more smaller cells in culture, due no doubt to the fact that during rapid development the buds separate when less fully developed than in the insect. The internal structure when stained, appears to be the same as that of the forms in the fat-body described above.

After prolonged incubation on solid media the formation of a distinct mycelium always occurs. This is at first white, but after several weeks, blackened spots sometimes become visible, due to the development of pigment in the walls of certain groups of cells. This occurs especially on potato-agar. In at least one liquid medium, Locke's solution, the same blackened cells develop.

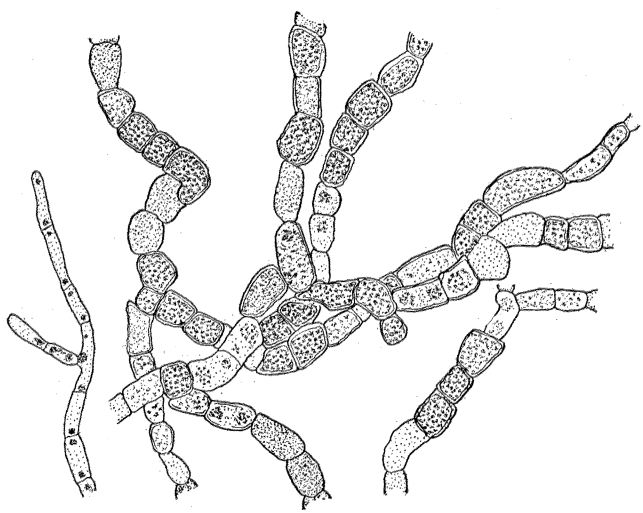

FIG. 2. Portion of mycelial growth of symbionts after prolonged incubation (10 days) in liquid bouillon medium. Magnified about 400 diameters.

The mycelium (Fig. 2) is branched and of quite irregular form. The larger hyphæ measure from 6–15 μ in diameter, broad and narrow cells frequently alternating or with one size interpolated in series of the other. Some cells, usually single ones or pairs, more rarely several in succession are heavily pig-

mented and appear very dark in fresh material; a pair of these are frequently rather closely fused to form an oval body. The contents of these dark cells are highly granular, with the protoplasmic mass clearly separated from the cell wall by a hyaline layer. Toward the tips the hyphæ are usually much more slender, pale in color and with only scattered granules in the protoplasm. The method of branching is entirely dichotomous, many lateral branches along the larger hyphæ consisting of but a single cell although both near the tips and on the larger hyphæ there are many long branches which again subdivide. Very rarely there is an anastomosis of the finer apical branches formed by lateral prolongations of the cells. In preparations we have had difficulty in recognizing the conidiophores, but free conidia are present in old cultures. They measure from 8–10 μ in length and are broadly oval in form, very nearly transparent and without color.

Systematic Position of the Cultivated Fungus.

We have been unable to deal with this matter in a satisfactory way owing to our unfamiliarity with cryptogamic botany and must leave it for consideration by an experienced mycologist. It is very evident that the symbiotic organism in *Pulvinaria* cannot be regarded as a Saccharomycete, although its morphology and method of multiplication in the insect does not preclude such an assumption. Indeed, the symbionts that have been observed in other Coccids and in most other Homoptera as well have usually been regarded as yeast-like organisms and commonly referred to the genus Saccharomyces or to new genera located in the same group of plants. Berlese ('06) has placed the organism which he cultivated from *Ceroplastes* in the genus *Oöspora,* thus recognizing it as a true fungus, but hitherto, with the possible exception of Pierantoni ('10) no one else seems to have been successful in growing *in vitro* any of the symbionts of coccids.

The species which we have obtained from *Pulvinaria* seems to be quite similar to the one described and figured by Berlese from *Ceroplastes* so far as the general morphology of the yeast-like cells in the coccid and the mycelial structure in culture. Neither species, however, has been sufficiently studied to make a more posi-

tive statement. Dr. O. F. Burger kindly examined some of our cultures and has expressed the opinion that they probably represent a species of *Dematium* or a related genus. Such morphological characters as we have been able to make out agree well with descriptions of this genus to which it may be tentatively referred.

Physiological Rôle of the Symbiont.

As stated at the outset, we have attempted to determine the physiological behavior of the *Pulvinaria* symbiont in culture to ascertain in what way it may affect the metabolism of the coccid.

Contrary to what occurs in the case of most yeasts, this organism produced no gas in media made from any of the sugars in which it was grown. This is quite what might be expected as the coccid tissues are undoubtedly rich in sugars and any organism producing gas in the presence of such substances could not be tolerated in the body of the coccid.

On the other hand a diastatic ferment is produced in quite appreciable quantities. Whether this bears any relation to the metabolism of the coccid is not entirely clear. In the adipose tissue and body liquids, starch is probably not present to any considerable extent, although in the large quantities of plant sap ingested by the coccids there must be substances upon which this ferment might act. It has been shown also by Büsgen ('91) that certain modifications are produced in the tissues of the food plants of Coccids at the point where the mouth setæ are thrust into the plant. These modifications appear to be induced by secretions actually injected into the plant tissue by the insects and they may act in liquefying or in partly digesting already liquid or semi-liquid material, before it is withdrawn by the insect. It is quite possible therefore that a diastatic ferment might act in two possible ways in aiding the digestion of the coccid. If freed in the blood, it might either pass into the alimentary tract, there to act upon ingested food, or it might be taken up by the salivary glands to be later injected into the plant and thus act as an extra-intestinal digestive agent. Such extra-intestinal digestion is known to occur in several diverse insects, although in these cases the ferments are no doubt elaborated directly by the salivary glands.

We have also clearly shown that a proteolytic enzyme and a lipase are produced abundantly by the *Pulvinaria* symbiont. The possibilities for these to influence the digestion and metabolism of the coccid are more diverse than those presented by the presence of diastase. Both, particularly the lipase, must act upon the adipose cells in which the symbionts occur. We might therefore suppose that they assist in the rapid breaking down of this tissue at the time of maturity when the eggs of the *Pulvinaria* are rapidly developed. That they may assist in digestion, either in the body or through the agency of secretions injected into the plant is also quite possible, although such indirect action must undoubtedly be secondary if it occurs at all.

The General Nature of the Relation between Symbiont and Coccid.

The symbionts have gradualy come to be regarded rather generally as truly symbiotic organisms, although those who first studied them naturally assumed that their presence indicated some sort of parasitism. There are several reasons why it is difficult to believe that they are actually parasitic. In the first place, not only in *Pulvinaria,* but in the other species in which they have been found, they are universally present in all the individuals of a species in approximately equal numbers. Many true parasites, *e.g.,* certain Nematode worms, the Protozoan parasites of human malaria, etc., commonly appear with great frequency in the bodies of their hosts, but their occurrence never includes all the individuals of a host species, except at certain times and places where parasitism is unusually heavy and assumes the form of an epidemic. In such cases also, the affected population is not in a healthy condition and species so generally affected cannot be expected to represent ones well fitted to survive and become abundant. *Pulvinaria* and other Coccids certainly cannot be placed in such a category. On the other hand the presence of detrimental parasites results in tissue changes or disturbances of metabolism that can be recognized. Such can be seen in the behavior of the fat-body in *Pulvinaria* and other genera (Sulc, '11), but as has just been said this is most readily regarded as beneficial rather

than detrimental as the fat is not broken down until the time that it would normally disintegrate to supply nourishment for the developing eggs of the coccids.

Without any definite indication of pathological changes, it seems impossible, therefore, to regard the universally present symbionts as harmful parasites.

It has also been suggested that they may represent innocuous or indifferent parasites and it is not so easy to distinguish between these and true symbiotic or benign organisms from their effect on the coccids. As a matter of fact it seems necessary to regard all three as steps in an evolutionary process, harmful parasites in their first association, later as innocuous ones and finally as true symbionts. These will follow one another as the host adapts itself to withstand or nullify any ill effects of the parasite until it finally is able to utilize the products of the intruder to further its own metabolic processes.

Thus it seems reasonable to regard these three types of association as not clearly distinct from one another, but as connected by intergrades.

Since, however, there is good reason to believe that the production of diastase, protease and lipase by the symbionts may serve to benefit the coccids, the possibility of real symbiosis cannot be excluded.

There is one point, however, which needs further study. By a minute study of the changes in the tissue of the food-plant adjacent to the proboscis of the feeding Coccid, it should be possible to gain much additional evidences upon the changes which undoubtedly occur in such tissue. This we have not had opportunity to undertake. Why the disintegration of the fat-body is delayed till the proper time in the life-cycle of the coccid also is not clear. Since, however, changes in the vegetative character of the symbiotic fungus are initiated in the late spring, it seems probable that they may determine to some extent the quantities of enzymes produced. On the other hand it is evident that the coccid is able to inhibit any excess development of the symbiont as the number of symbiont cells remains very uniform and never seems to increase beyond certain bounds, quite a different condi-

tion from that obtained among pathogenic parasitic microörganisms.[1]

This indicates a nice physiological balance between the coccid and symbiont and is another reason for considering this a case of true mutualism.

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[1] In this connection it is interesting to note that we found antibodies developed abundantly in rabbits immunized against our cultures of the *Pulvinaria* symbionts.

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EXPLANATION OF PLATES.

PLATE I.

1. Cross-section of overwintering Coccid, showing distribution of symbionts in the fat-body. Low magnification.

2. Portion of fat-body at higher magnification with symbionts included in adipose cells. Magnification about 400 diameters.

3. Similar portion, showing more abundant symbionts. Magnification about 400 diameters.

4. Symbionts in adipose tissue which is apparently in process of disintegration. Magnification about 400 diameters.

5. Smear from culture of symbionts in liquid medium after 48 hours incubation. Magnification about 400 diameters.

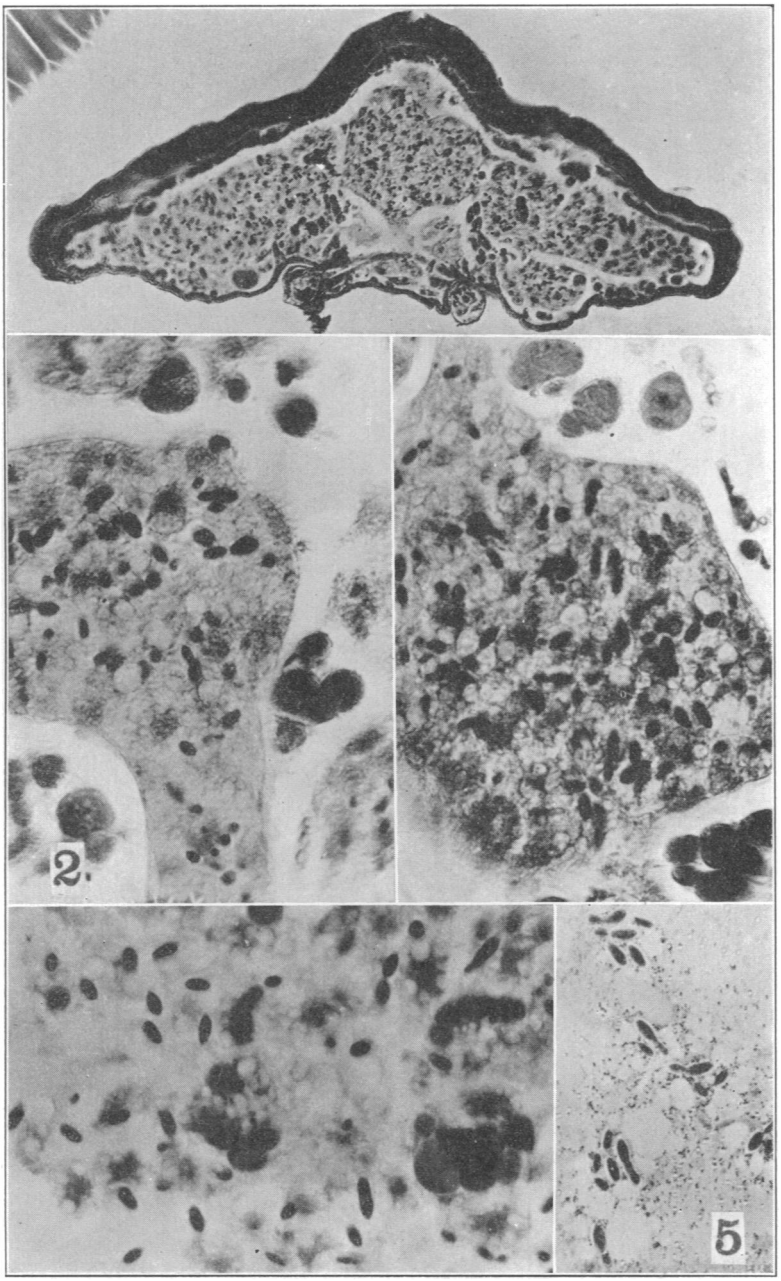

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PLATE II.

6. Form of young colonies of symbiont after 72 hours incubation on potato agar plate, viewed by reflected light. Magnified 5 diameters.

7. Group of colonies of symbiont on nutrient agar after 10 days incubation. Magnified 4 diameters. Note small size of colonies and paucity of processes.

8. Group of colonies on the plate illustrated in figure 6, at same magnification, viewed by transmitted light, to show internal structure and manner in which radial processes develop.

9. Development of mycelia in liquid culture of symbiont after prolonged incubation of 10 days. Magnified about 60 diameters.

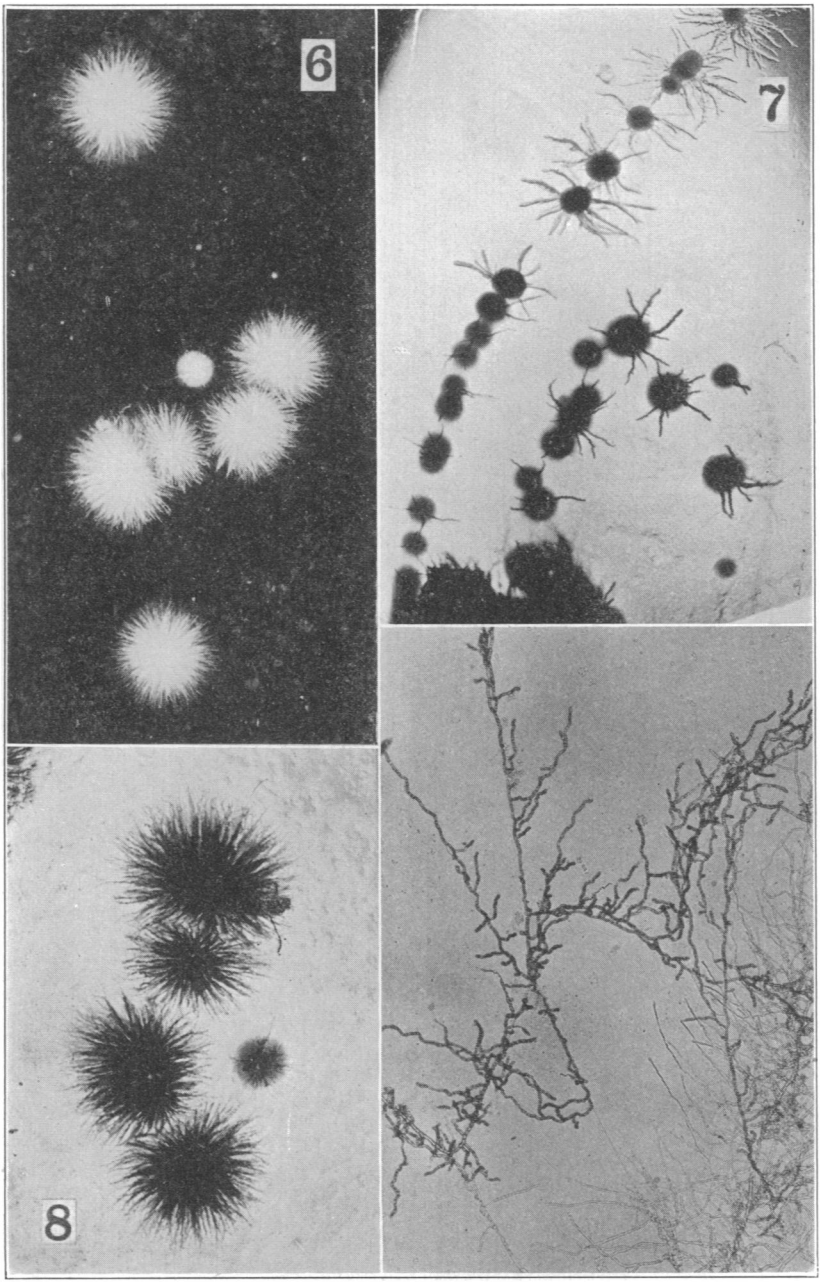

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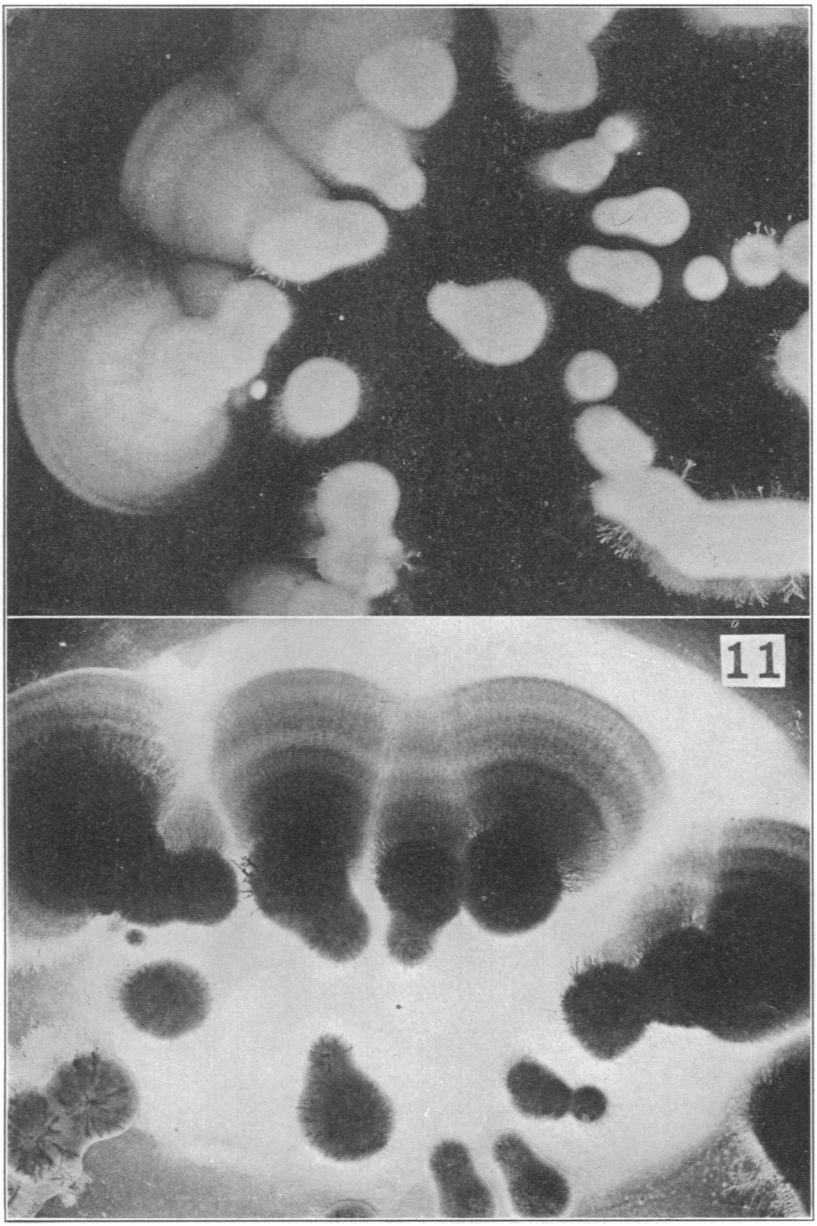
PLATE III.

10. Gross appearance of colonies of symbiont after prolonged incubation (12 days) on potato agar. Viewed by reflected light to show peripheral processes. Magnified 3 diameters.

11. Portion of same plate at same magnification, viewed by transmitted light, to show internal mycelial structure.



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